Condensed title: ARF6-JIP3/4 axis controls breast tumor invasion
ABSTRACT

Invasion of cancer cells into collagen-rich extracellular matrix requires membrane-tethered (MT)1-MMP as the key protease for collagen breakdown. Understanding how MT1-MMP is delivered to the surface of tumor cells is essential for cancer cell biology. Here we identify ARF6 together with JIP3 and JIP4 effectors as critical regulators of this process. Silencing ARF6 or JIP3/JIP4 in breast tumor cells results in MT1-MMP endosome mispositioning and reduces MT1-MMP exocytosis and tumor cell invasion. JIPs are recruited by WASH on MT1-MMP endosomes on which they recruit dynein/dynactin and kinesin-1. Interaction of plasma membrane ARF6 with endosomal JIPs coordinates dynactin/dynein and kinesin-1 activity in a tug-of-war mechanism leading to endosome tubulation and MT1-MMP exocytosis. In addition we find that ARF6, MT1-MMP and kinesin-1 are up-regulated in high-grade triple-negative breast cancers. These data identify a critical ARF6/JIP/MT1-MMP/dynein-dynactin/kinesin-1 axis promoting an invasive phenotype of breast cancer cells.
INTRODUCTION

The ability of tumor cells to invade surrounding tissue and disseminate to distant sites is one hallmark of cancer and a predominant cause of cancer-related death. One intrinsic property of metastatic tumor cells is their ability to degrade components of the extracellular matrix (ECM) and thereby to breach tissue barriers. ECM remodeling by cancer cells is executed by matrix-degrading proteases (Bonnans et al., 2014). Membrane-anchored membrane type 1 matrix metalloproteinase (MT1-MMP) is overexpressed by carcinoma cells of various origins and is a critical mediator of pericellular matrix remodeling required for invasive tumor growth and metastasis (Hotary et al., 2006; Hotary et al., 2003; Lodillinsky et al., 2015).

Surface levels of MT1-MMP increase during breast tumor progression particularly in targeted therapy-lacking triple-negative breast cancers (TNBCs) (Lodillinsky et al., 2015). In TNBC cell lines, newly synthesized MT1-MMP reaches the plasma membrane and is rapidly internalized (Poincloux et al., 2009). Internalized MT1-MMP accumulates in late endocytic compartments from where it is delivered to invadopodia, corresponding to specialized plasma membrane/matrix contact sites involved in pericellular matrix proteolysis (Hoshino et al., 2013; Monteiro et al., 2013; Steffen et al., 2008; Williams and Coppolino, 2011; Yu et al., 2012). Delivery of MT1-MMP to invadopodia requires tubular membrane connections forming between MT1-MMP-containing late endosomes and the invadopodial plasma membrane (Monteiro et al., 2013). This mechanism requires MT1-MMP-containing endosomes to be transported to the cell periphery towards invadopodia (Monteiro et al., 2013; Steffen et al., 2008; Yu et al., 2012). Along this line, trafficking of MT1-MMP involves microtubules and microtubule plus(→)-end-directed kinesin motors in human macrophages (Wiesner et al., 2010).
Late endosomes exhibit bidirectional motility as a result of a tug-of-war between dynein/dynactin and kinesin motors of opposite direction (Granger et al., 2014). The direction of endosome movement can be controlled by motor adaptor proteins including JNK interactor proteins 3 and 4 (JIP3 and JIP4), which bind to kinesin-1 and to dynactin (Bowman et al., 2000; Cavalli et al., 2005; Montagnac et al., 2009; Sun et al., 2011). Switching of JIP3/JIP4 between kinesin-1 and dynactin/dynein on recycling endosomes is regulated by the small GTPase ARF6, which binds JIP3/JIP4 in its GTP-bound activated form (Montagnac et al., 2009). A large body of work implicates ARF6 in the motile phenotype and metastatic potential of cancer cells (D'Souza-Schorey and Chavrier, 2006). Overexpression of ARF6 correlates with increased matrix invasion activity of melanoma and breast tumor-derived cell lines (Hashimoto et al., 2004; Tague et al., 2004). A pathway consisting of ARF6, the ARF6 guanine exchange factor (GEF) GEP100/BRAG2 and AMAP1 (DDEF1 or ASAP1), an ARF6 downstream effector, promotes tumor invasion and metastasis in breast cancer in response to epidermal growth factor receptor (EGF-R) activation (Morishige et al., 2008; Sabe et al., 2009).

Here, we analyzed the contribution of ARF6 and JIP3/JIP4 effector proteins to the trafficking of MT1-MMP in breast cancer cells. We found that JIP3/JIP4 control the recruitment of dynactin/dynein and kinesin-1 motor proteins on MT1-MMP-positive endosomes, while kinesin-2 recruitment is independent of JIP. Through interaction with endosomal JIP3/JIP4, plasma membrane ARF6 opposes to dynactin/dynein-independent movement of MT1-MMP endosomes, promoting endosomal membrane tubulation by kinesin-1 and the transfer of MT1-MMP to the plasma membrane. JIP recruitment to MT1-MMP endosomes requires endosomal Arp2/3 complex activator WASH, suggesting coordination of actin-based tubular membrane deformation and
microtubule-dependent pulling force generation for endosomal membrane tubule formation. Immunohistochemistry analysis of invasive breast tumor specimens revealed a co-up-regulation of KIF5B kinesin-1 subunit, MT1-MMP and plasma membrane ARF6 in high grade TNBCs identifying MT1-MMP-ARF6-JIP3/JIP4-kinesin-1 axis in breast cancer invasion.
RESULTS

ARF6 is required for matrix remodeling and invasive migration by TNBC cell lines

ARF6 silencing was assessed in MDA-MB-231 cells, classified as highly invasive TNBC (Neve et al., 2006). Confirming earlier reports (Hashimoto et al., 2004; Tague et al., 2004), silencing of ARF6 by siRNAs (Fig. S1A and C) decreased FITC-gelatin degradation by 60% as compared to cells treated with non-targeting siRNA (siNT) (Fig. 1A). MT1-MMP knockdown almost completely abolished gelatin degradation by MDA-MB-231 cells (Fig. 1A and Fig. S1B). ARF6 knockdown did not interfere with MT1-MMP expression (Fig. S1A and C). We also investigated the consequence of ARF6 silencing on the capacity of breast cancer cells to invade in a 3D type I collagen matrix over a 2-day period. As compared to multicellular spheroids of MDA-MB-231 cells treated with siNT, invasion by ARF6-depleted spheroids was decreased by about 40% similar to MT1-MMP knocked-down spheroids (Fig. 1B-C and Fig. S1D).

The generality of ARF6’s contribution to the invasive potential was assessed in MCF10DCIS.com cells, which generate ductal carcinoma in situ (DCIS)-like lesions and require MT1-MMP for invasion (Hu et al., 2008; Lodillinsky et al., 2015). Similar to the situation in MDA-MB-231 cells, knockdown of ARF6 or MT1-MMP (Fig. S1E) led to 50% decrease of invasion capacity of MCF10DCIS.com multicellular spheroids in type I collagen (Fig. S1F-G). We then sought to investigate whether inhibition of cell invasion was due to a requirement for ARF6 in TNBC’s ability to proteolytically cleave surrounding collagen fibers. We used the neoepitope Col1-3/4C antibody, which recognizes collagenase-cleaved fragment of collagen I (Monteiro et al., 2013;
Wolf et al., 2007). In MDA-MB-231 cells embedded in type I collagen, depletion of MT1-MMP strongly reduced pericellular collagen degradation as indicated by 70% reduction of Col1-3/4C antibody signal (Fig. 1D-E). Thus, collagen degradation by MDA-MB-231 cells strongly relies on MT1-MMP activity. Silencing of ARF6 led to a similar decrease of collagen proteolysis (Fig. 1D-E), suggesting that ARF6 is implicated in MT1-MMP-dependent pericellular collagen degradation. All together, these data indicate a requirement for ARF6 in matrix remodeling and in the invasive potential of TNBC cells.

**JIP3 and JIP4 are required for invasive migration through 3D type I collagen**

We initially identified the related JIP3 and JIP4 proteins as ARF6 downstream effectors controlling microtubule-based endosome movement through regulation of kinesin-1 and dynactin/dynein complex (Isabet et al., 2009; Montagnac et al., 2009), suggesting possible role for ARF6-JIP3/JIP4 in microtubule-dependent movement of MT1-MMP-positive endosomes. JIP3 and JIP4 expression was detected by immunoblotting analysis in MDA-MB-231 cells (Fig. S2AB). Immunofluorescence staining (IF) revealed a cytosolic diffuse distribution and a punctate association with the cytosolic face of cytoplasmic vesicles; most of these vesicles were positive for MT1-MMPmCherry (MT1-MMPmCh) (Sakurai-Yageta et al., 2008a)(Fig. 2A). Staining was abolished by JIP4 silencing (Fig. S2C). JIP3 could not be detected due to lack of IF-grade antibodies. The contribution of JIP3 and JIP4 to the invasive potential of MDA-MB-231 cells was analyzed. Knockdown of JIP3 or JIP4 led to significant decrease of FITC-gelatin degradation (Fig. S2D) and invasion of multicellular spheroids in 3D type I collagen (Fig. 2B and Fig. S2E). Double
knockdown of JIP3 and JIP4 using two independent pairs of siRNAs resulted in a similar inhibition of invasion suggesting mutually dependent function of the two proteins (Fig. 2C and Fig. S2F). Moreover, the requirement for JIP3 and JIP4 in invasive migration was paralleled by a 50-65% decrease in the capacity of JIP3/JIP4-depleted cells to cleave type I collagen (Fig. 2D and Fig. S2G). Thus, together with ARF6, our data identify JIP3 and JIP4 as important components of the matrix remodeling and invasion program of MDA-MB-231 breast tumor cells.

The ARF6/JIP pathway controls MT1-MMP-positive endosome positioning and exocytosis

VAMP7-, Rab7-positive late endosomes (LEs) represent a major reservoir of MT1-MMP involved in exocytosis and surface delivery of the protease to support pericellular matrix proteolysis (Monteiro et al., 2013; Steffen et al., 2008; Williams and Coppolino, 2011; Yu et al., 2012). The capacity of ARF6 and JIPs to control the distribution of MT1-MMP-positive LEs was assessed. Automated image analysis of endosome position showed that MT1-MMPmCh accumulated in large centrally located endosomes and smaller, more dispersed endosomal compartments (siNT, Fig. 2E, blue curve and Fig. 2F) with strong overall overlap with Rab7 late endocytic marker (Fig. S3A and C) (Monteiro et al., 2013; Steffen et al., 2008). Analysis of vesicle movement by color-coding maximum intensity time projection of selected frames from time-lapse sequences revealed highly dynamic bidirectional movement of MT1-MMP-positive endosomes (Fig. 2F, inset 1 and Movie S1). Silencing of ARF6 with three independent siRNAs led to a dramatic redistribution and clustering of MT1-MMP-positive endosomes to the central cell region (Fig. 2E, orange-brown curves...
and Fig. 2F) where these large vacuolar structures remained essentially static (appearing as white in color-coded representation of movement, Fig 2F, inset 2 and Movie S1). On the opposite, JIP3/JIP4 double-knocked down cells had a more dispersed distribution of MT1-MMP endosomes (Fig. 2E, green curve) and accumulated clusters of MT1-MMP-positive vesicles at the cell periphery, which were mostly non motile (Fig 2F and insets 3 to 5 and Movie S1). Knockdown of ARF6 or JIP did not affect MT1-MMP and Rab7 overlap (Fig. S3A and C) nor limited co-localization with EEA1-positive early endosomal compartments (Fig. S3BC), indicating that loss of ARF6 or JIP function affected the steady-state distribution of Rab7/MT1-MMP-positive LEs with minimal effect on endosomal cargo sorting and/or endosome maturation. The amino-terminal region of JIP3 and JIP4 contains two leucine zipper (LZI and LZII) domains known to mediate dimerization of the proteins (Isabet et al., 2009; Kelkar et al., 2000; Nguyen et al., 2005). As an alternative approach to interfere with JIP3/JIP4 dimerization and inhibit their function we overexpressed the LZ1 domain of JIP3 (amino acid 1-266). JIP3-LZI induced the redistribution of MT1-MMP endosomes to the cell periphery similar to JIP3/JIP4 silencing (Fig. 2G, light green). In contrast, expression of GFP or a variant JIP3-LZI domain with two Leucine-to-Proline substitutions (LZI/L83P-L89P) abolishing dimerization (not shown) did not affect MT1-MMP localization (Fig. 2G, dark green).

As surface MT1-MMP is directly responsible for pericellular matrix degradation, we investigated the role of ARF6 and JIP3/JIP4 in MT1-MMP exocytosis from LEs in relation with their identified function in endosome positioning. MDA-MB-231 cells expressing MT1-MMPpHLuorin were cultured on fibrillar type I collagen and we monitored apparition of green fluorescence flashes corresponding to de-quenching of the fluorescence of extracellular pHLuorin tag upon exocytosis of MT1-MMP-positive
LEs (Monteiro et al., 2013). As previously reported (Monteiro et al., 2013), exocytic events occurred mainly in association with collagen I fibers and led to surface accumulation of MT1-MMP along ECM fibers (Fig. S3D). Quantification of MT1-MMPpHluorin flashes revealed a decrease in frequency of MT1-MMP exocytic events upon ARF6 or JIP3/4 knockdown (Fig. S3E). Therefore, mispositioning of MT1-MMP-containing LEs as a consequence of ARF6 or JIP3/JIP4 loss-of-function correlated with impaired MT1-MMP exocytosis. All together, we conclude that defects in matrix remodeling and invasive migration correlate with dramatic and opposite changes in the distribution of MT1-MMP-positive LEs in MDA-MB-231 cells depleted for ARF6 or JIP3/JIP4, respectively. These effects culminate in decrease in MT1-MMP exocytosis at the surface and inhibition of pericellular matrix degradation and correlate with reduced invasive potential of TNBCs.

**Regulation of MT1-MMP endosome position by ARF6 requires JIP3/JIP4 and p150\textsuperscript{Glued} dynactin complex subunit**

Perinuclear clustering of MT1-MMP-positive endosomes could indicate unbalanced dynactin/dynein minus(-)-end directed motor activity in ARF6-depleted MDA-MB-231 cells. Along this line, knockdown of the dynactin complex subunit p150\textsuperscript{Glued} (Fig. S3F) induced a more dispersed and peripheral distribution of MT1-MMP endosomes (Fig. 2H, pink curve). In epistasis experiments, p150\textsuperscript{Glued} knockdown restored a normal-like distribution of MT1-MMP endosomes in ARF6-depleted cells as compared to perinuclear clustering in cells depleted for ARF6 only (Fig. 2F and H, compare light blue vs. orange curve and Fig. S3FG). Thus, loss of ARF6 seems to favor dynein function. Similarly, depletion of ARF6 and JIP3/JIP4 together also reverted si-ARF6-
mediated perinuclear MT1-MMP endosome clustering and led to the accumulation of endosomes at the cell periphery (Fig. 2F and H, compare brown vs. orange curve), indicating a requirement for JIP3/JIP4 function in regulation of endosome positioning by ARF6 through control of dynein/dynactin complex function. Finally, double p150Glued and JIP3/JIP4 knockdown resulted in a scattered and peripheral distribution of MT1-MMP-positive endosomes as in the single knockdown of each protein (Fig. 3H, compare brown vs. green or pink curve and Fig. S3H), supporting the conclusion that JIPs and dynactin complex work within the same pathway.

Kinesin-1 and kinesin-2 are required for MT1-MMP endosome movement

Association of kinesin-1 (KIF5B), kinesin-2 (KIF3A) and p150Glued dynactin complex subunit with MT1-MMP-containing endosomes was assessed at endogenous level by double-labeling IF analysis and 3D deconvolution microscopy. Specificity of antibodies to p150Glued, KIF5B and KIF3A was demonstrated by loss of staining in cells silenced for these proteins (Fig. S4A). MDA-MB-231 cells stably expressing MT1-MMPmCh were double labeled for JIP4 and KIF5B or p150Glued. As expected for motors having pleiotropic associations with various cell organelles, KIF5B and p150Glued showed a cytoplasmic dotty distribution with some association with the cytosolic face of MT1-MMPmCh-containing endosomes decorated by JIP4 puncta (Fig. 3AB). Some co-localization was visible between JIP4 and p150Glued (Fig. 3B, arrows), which was confirmed by in situ proximity ligation assay (PLA) (Fig. 3EF). PLA signal was strongly reduced upon JIP3/JIP4 knockdown or when JIP4 or p150Glued antibody was omitted (Fig. 3F). All together, these observations indicate a close proximity between JIP4 and p150Glued on MT1-MMP-positive compartments. In
addition, several cytoplasmic puncta of KIF3A were detected in association with Rab7-, MT1-MMPmCh-positive LEs (Fig. 3C). Double labeling for KIF3A and KIF5B showed that both kinesins were associated with the same MT1-MMP-positive endosomes (Fig. 3D).

KIF5B-YFP and KIF3A-GFP subunits were stably expressed in MDA-MB-231 cells at levels similar to the endogenous subunits (Fig. S4BC). The distribution of MT1-MMPmCh-positive endosomes was more dispersed and peripheral in cells expressing KIF5B-YFP or KIF3A-GFP as compared to control cells expressing YFP (Fig. S4DE). Color-coding of vesicle movement showed that MT1-MMP endosomes retained both anterograde and retrograde motility (Fig. S4D, lower panels). To quantitatively assess differences in motility of MT1-MMPmCh-positive LE in cells stably expressing KIF5B- or KIF3A as compared to control YFP-expressing cells in an unbiased manner, we calculated a displacement index for each cell population (Quintero et al., 2009, see Materials and Methods section). The displacement index increased ~2.3-fold in cells stably expressing KIF5B- or KIF3A as compared to control YFP-expressing cells (Fig. S4F). In contrast, silencing of KIF5B or KIF3A in MDA-MB-231 cells led to a ~2-fold reduction of overall endosome motility (Fig. S4G).

All together, these results indicate that MT1-MMP-positive endosome motility relies on association and activity of kinesin-1 and -2 and confirm the role of p150Glued dynactin subunit in (-)-end directed movement of MT1-MMP-positive LEs in breast cancer cells.

**JIP3/JIP4 regulate motor association on MT1-MMP endosomes**
Given ARF6 and JIP3/JIP4’s roles in MT1-MMP endosome positioning and interaction with kinesin-1 and dynein/dynactin complex, we investigated potential implication of ARF6 and JIPs on endosomal association of KIF5B, KIF3A and p150\textsubscript{Glued} using two independent approaches. First, Arf6 or JIP3/JIP4 were silenced and after fixation and IF staining for motor proteins, MT1-MMP\textsubscript{mCh}-positive compartments were segmented from deconvoluted microscopy images and number of puncta of motor proteins associated with each segmented MT1-MMP-positive vesicle was scored (Fig. 3GH). Using this approach, we found that JIP3/JIP4 silencing correlated with a 50-60% decrease of KIF5B and p150\textsubscript{Glued} association with MT1-MMP-positive endosomes, while KIF3A association was not affected (Fig. 3GH). The effect of ARF6 knockdown was more difficult to analyze as loss of ARF6 function led to clustering and various extent of MT1-MMP endosome collapse in the cell center (see Fig. 2F). Consequently, motor association values were highly variable for all three motor proteins and increased non-significantly probably as a consequence of endosome enlargement (Fig. 3GH).

In addition, association of motor proteins with MT1-MMP cargo was analyzed by co-immunoprecipitation experiments. KIF5B and KIF3A co-immunoprecipitated with MT1-MMP expressed at endogenous level in MDA-MB-231 cells (Fig. 3I). In addition, overexpressed MT1-MMP\textsubscript{mCh} co-immunoprecipitated with GFP-tagged KIF5B or KIF3A (Fig. 3J). In reciprocal experiments endogenous KIF5B, p150\textsubscript{Glued} or KIF3A co-immunoprecipitated with overexpressed MT1-MMP\textsubscript{mCh} in control siNT-treated cells (Fig. 3KL). Silencing of JIP3/JIP4 significantly decreased MT1-MMP association with KIF5B and p150\textsubscript{Glued} proteins while having no effect on MT1-MMP/KIF3A interaction (Fig. 3KL). Noticeably, knockdown of ARF6 did not interfere with association of MT1-MMP with KIF5B, p150\textsubscript{Glued} or KIF3A (Fig. 3KL). Thus our data are consistent with
JIP3/JIP4 controlling the transport and dynamics of MT1-MMP storage endosomes through the association of KIF5B/kinesin-1 and dynactin complex with MT1-MMP endosomes while having no effect on KIF3A/kinesin-2 recruitment.

Finally, we investigated whether KIF3A/kinesin-2 may be responsible for the peripheral distribution of MT1-MMP endosomes observed in cells silenced or inhibited for JIP3/JIP4 (Fig. 2EF) as a consequence of kinesin-1 and dynactin loss (Fig. 3GH and KL). Triple silencing of JIP3/JIP4 and KIF3A changed the distribution of MT1-MMP-positive endosomes, which appeared as clusters scattered in the cytoplasm (Fig. 4A-C, yellow curve), different from their peripheral distribution in JIP3/JIP4 double-depleted cells (green curve) or perinuclear one in KIF3A-single depleted cells (purple curve). Analysis of vesicle movement revealed that MT1-MMP endosome clusters in JIP3/JIP4-KIF3A-triple depleted cells were mostly immobile similar to the situation observed in nocodazole-treated cells and consistent with motorless MT1-MMP endosomes (Fig. 4D-F and Movie S2). Thus kinesin-2 redistributes MT1-MMP endosomes to the cell periphery in JIP3/JIP4 deficient cells.

**ARF6, JIP3/JIP4 and p150<sup>Glued</sup> are required for KIF5B-mediated MT1-MMP endosome tubulogenesis**

Functional consequences of kinesin-mediated movement of MT1-MMP-containing endosomes on the invasive potential of breast tumor cells were assessed. Silencing of KIF3A or KIF5B with independent siRNA pairs (Fig. 5A-C) resulted in strong reduction of MDA-MB-231 cells’ ability to degrade FITC-gelatin and to invade a 3D type I collagen matrix in a circular invasion assay, similar to MT1-MMP knockdown (Fig. 5DE). Similar effects were observed upon silencing of KIF5B or KIF3A in
MCF10DCIS.com cells (Fig. 5FG). In addition, silencing of KIF3A or KIF5B decreased pericellular type I collagen proteolysis in association with a reduction of MT1-MMPpHLuorin exocytosis (Fig. 5HI).

We then focused our analysis on KIF5B whose association with MT1-MMP endosomes is regulated by JIP3/JIP4. ARF6 silencing shifted the scattered distribution of MT1-MMP-positive compartments in KIF5B-overexpressing cells to the perinuclear region, confirming that loss of ARF6 function strongly promoted (-)-end dynactin/dynein complex-dependent movement dominantly over KIF5B overexpression (Fig. 6AB, compare brown and blue curves). As already observed in parental MDA-MB-231 cells (Fig. 2EF), knockdown of JIP3/JIP4 triggered a peripheral distribution of MT1-MMP endosomes (Fig. 6AB, green and blue curves) probably dependent on KIF3A (see Fig. 4). Similarly, p150Glued knockdown resulted in a shift of endosome distribution towards the cell periphery likely as a consequence of loss of kinesin-antagonizing activity of the dynactin/dynein complex (Fig. 6AB, pink and blue curves).

Next, we investigated the matrix degradative capacity of the different KIF5B-overexpressing cell populations. Correlating with increased dynamics and peripheral distribution of MT1-MMP-positive endosomes (Fig. S4), overexpression of KIF5B resulted in a dramatic 3.5-fold up-regulation of FITC-gelatin degradation capacity (Fig. 6C). As in parental MDA-MB-231 cells (Fig. 1A), loss of ARF6 function causing perinuclear accumulation of MT1-MMP endosomes abolished matrix-degradation promoting effect of KIF5B overexpression (Fig. 6C and Fig. S5A and C). More surprising was the observation that JIP3/JIP4 loss of function also interfered with gelatin-degradation by KIF5B-overexpressing cells despite a pronounced peripheral distribution of MT1-MMP-positive endosomes (Fig. 6C and Fig. S5A and C).
Similarly, \(p150^{\text{Glued}}\) knock down affected matrix degradation by KIF5B-overexpressing cells (Fig. 6C and Fig. S5BC). Thus like in MDA-MB-231 parental cells, loss of ARF6 or JIP3/JIP4 function although having opposite effects on MT1-MMP endosome distribution resulted in similar impairment of pericellular matrix degradative activity of MDA-MB-231, which could not be compensated for by KIF5B overexpression and/or endogenous KIF3A activity.

Surface delivery of MT1-MMP involves formation of tubular connection between storage endosomes and the plasma membrane in association with ECM fibers (Monteiro et al., 2013; Steffen et al., 2008). Close inspection of time-lapse movies of MT1-MMPmCh endosomes in cells overexpressing KIF5B revealed highly dynamic tubular structures forming from MT1-MMP-positive vesicular compartments in the vicinity of the ventral plasma membrane in contact with the matrix (Fig. 6D, siNT and Movie S3). Time-lapse TIRFM analysis confirmed that tubulogenesis from MT1-MMP endosomes took place in association with the plasma membrane (Fig. 6E). Knockdown of JIP3/JIP4 diminished tubulogenesis of MT1-MMP endosome in the vicinity of the ventral cell surface by 2-fold (Fig. 6DF and Movie S3). This finding suggests that KIF3A activity, which is responsible for peripheral accumulation of MT1-MMP-positive compartments at the microtubule (+)-end in JIP3/JIP4-depleted cells (Fig. 4) does not support tubulogenesis. Along this line, KIF3A silencing did not significantly affect tubulogenesis (Fig. 6DF). In addition, silencing of \(p150^{\text{Glued}}\) inhibited endosomal tubule formation indicating that dynactin function is also required for tubulogenesis from MT1-MMP-positive compartments (Fig. 6DF and Movie S3). Of note, tubule formation was not detected from perinuclear clustered MT1-MMP endosomes in ARF6-depleted cells (not shown). Tubulogenesis was also visible in MDA-MB-231 cells with endogenous kinesin-1 level although to lower extent (not
shown and see (Monteiro et al., 2013; Steffen et al., 2008)) and was diminished by 2-fold upon JIP3/JIP4 knockdown (Fig. 6G)(Monteiro et al., 2013; Steffen et al., 2008). Thus we conclude that impaired MT1-MMP exocytosis and pericellular matrix remodeling upon knockdown of ARF6, JIP3/JIP4 or p150^Glued^ dynactin complex subunit correlate with decreased tubulogenesis capacity of MT1-MMP-positive endosomes in association with subplasmalemma microtubules.

**WASH is required for JIP4 association and function on MT1-MMP endosomes**

So far our data pointed to an essential role of tubulogenesis of peripheral MT1-MMP storage endosomes for MT1-MMP targeting to the plasma membrane based on a mechanism requiring ARF6 and JIP3/JIP4 controlling KIF5B/kinesin-1 and p150^Glued/^dynactin proteins. We reported earlier that formation of tubular connections between MT1-MMP-containing LEs and the plasma membrane and MT1-MMP exocytosis required the WASH complex (Monteiro et al., 2013). The punctate staining of JIP4 (Fig. 2A) was reminiscent of WASH and Arp2/3 complex/cortactin/F-actin distribution on MT1-MMP endosomes (Monteiro et al., 2013; Rosse et al., 2014). Thus we compared the localization of JIP4 and cortactin in MDA-MB-231 cells and found a close apposition of puncta of the two proteins on the cytosolic face of MT1-MMPmCh-positive endosomes (Fig. 7A). Moreover, WASH knockdown led to a strong reduction of cortactin puncta on MT1-MMP endosomes paralleled by an even stronger loss of JIP4 (Fig. 7AB and Fig. S5D). Thus, JIP4 association to MT1-MMP endosomes requires WASH. Similar to the effects of JIP3/JIP4 depletion, silencing of WASH in MDA-MB-231 cells overexpressing KIF5B decreased tubulogenesis of peripherally located MT1-MMP endosomes and strongly inhibited gelatin degradation.
capacity of the cells (Fig. 7CDE and Fig. S5E). All together, our data suggest that ARF6 and JIP3/JIP4 control a tug-of-war mechanism of kinesin-1 and dynactin/dynein involved in endosome tubulogenesis possibly in conjunction with WASH-, F-actin-based endosome membrane deformation required for MT1-MMP exocytosis in breast cancer cells (see discussion below and model in Fig. 7FG).

**ARF6, MT1-MMP and KIF5B up-regulation in TNBCs**

Data on ARF6 expression in breast cancers have been missing because of lack of suitable ARF6 antibodies for immunohistochemistry (IHC) of clinical specimens. One monoclonal anti-ARF6 antibody was selected and its specificity validated based on loss of IHC signal on section of multicellular spheroids of MCF10DCIS.com cells knocked down for ARF6 expression (not shown). Changes in ARF6 levels in breast carcinoma cells and association with breast cancer markers were investigated by IHC analysis of a tissue microarray (TMA) of invasive ductal carcinomas (IDCs) (characteristics of 496 patients summarized in Table S1 and see (Lodillinsky et al., 2015)). ARF6 staining was diffuse and cytosolic in luminal epithelial cells from peritumoral breast epithelial tissues (Fig. 8A and Fig. 10B), and was also detected in myoepithelial and stromal cells including fibroblasts and immune cells (not shown). Based on analysis of 426 IDCs available for scoring and using a H-score method (intensity score x % of positive cells), levels of cytosolic ARF6 were significantly higher in carcinoma cells as compared to adjacent epithelial cells in peritumoral tissues, while there was no significant difference between in situ and invasive components of IDCs (Fig. 8AB). Strikingly, ARF6 was present at cell-cell contacts in a subset of tumors (107/426, 25.1%) (Fig. 8A and Fig. 10B). Membranous ARF6
staining was restricted to carcinoma cells and never detected in normal breast epithelial cells in peritumoral tissues and was significantly higher in invasive vs. in situ components of IDCs, particularly in TNBCs (Fig. 8C and Fig. 9F).

These data indicate that ARF6 is up-regulated during breast tumor progression and accumulates at the plasma membrane of carcinoma cells in IDCs, possibly as a result of hyperactivation (Morishige et al., 2008). We previously reported that MT1-MMP levels increased at the surface of carcinoma cells in TNBCs based on IHC analysis of the TMA of human IDCs (Lodillinsky et al., 2015). A similar analysis was carried out for KIF3A and KIF5B to test the hypothesis that ARF6 may cooperate with motor proteins during MT1-MMP-dependent cell invasion in invasive breast cancers. The semi-quantitative H-score method was used for the four markers based on specific staining patterns, i.e. membranous ARF6 and MT1-MMP and total KIF3A and KIF5B (see Fig. 9A-D and Fig. 10A-D). For all four markers, levels were significantly higher in cancer cells as compared to mammary epithelial cells in peritumoral tissues (Fig. 9E). Based on 311 IDCs available for scoring, levels of membranous ARF6, surface MT1-MMP and KIF5B were positively correlated (Table S2AB) and significantly increased in high-grade (grade 3) tumors; all three markers were higher in estrogen receptor (ER)- and progesterone receptor (PR)-negative tumors, particularly in TNBCs (Fig. 9FG). In contrast, KIF3A levels negatively correlated with ARF6 and MT1-MMP expression (Table S2AB) and were higher in lower grades and in ER- and PR-positive (luminal) tumors (Fig. 9FG). A hierarchical clustering algorithm was applied for analysis of H-scores. ARF6, MT1-MMP and KIF5B co-clustered in their staining patterns, while KIF3A segregated away (Fig. 10E). Hierarchical clustering analysis showed that the 311 IDCs segregated into two main branches; one of the branch comprised a subgroup of 30 IDCs with strong staining
for ARF6, MT1-MMP and KIF5B markers and low KIF3A levels corresponding mostly to grade 3 (27/30) TNBCs (20/30) (Fig. 10E). Collectively, these data suggest interplay of ARF6, KIF5B and MT1-MMP in aggressive high-grade TNBCs.
DISCUSSION

ARF6 was shown early on to localize to invadopodia, specialized matrix-degradative structures of tumor cells and to be required for invadopodia activity (Hashimoto et al., 2004; Tague et al., 2004). Pioneering work identified an ARF6 pathway, in which ARF6 activated by the guanine nucleotide exchange factor GEP100 at the plasma membrane is pivotal for invasion and metastasis of breast tumor-derived cell lines (Hashimoto et al., 2006; Morishige et al., 2008; Onodera et al., 2005; Onodera et al., 2012). Here, we found that ARF6, MT1-MMP and KIF5B kinesin-1 heavy chain subunit are abnormally overexpressed in high-grade TNBCs. To our knowledge this is the first study reporting on dysregulation of ARF6 protein levels and correlation with MT1-MMP in breast tumor specimens. Up-regulation of ARF6, MT1-MMP and KIF5B in TNBCs is pathologically highly relevant given correlation between MT1-MMP levels and tumor progression and increased metastatic potential in this subgroup of highly aggressive breast cancers (Lodillinsky et al., 2015; Perentes et al., 2011).

MT1-MMP is targeted to endosomal compartments following endocytosis from the cell surface and then is recycled mainly from late endocytic storage compartments back to the surface of invasive TNBC cells (Monteiro et al., 2013; Steffen et al., 2008; Williams and Coppolino, 2011; Yu et al., 2012). Exocytosis of MT1-MMP occurs at invadopodia forming at matrix contact sites where MT1-MMP is essential for remodeling specific pericellular ECM substrates required for transmigration of cancer cells through the basement membrane, locoregional invasion and tumor growth and metastasis (Artym et al., 2006; Hoshino et al., 2013; Hotary et al., 2006; Hotary et al., 2003; Hu et al., 2008; Lodillinsky et al., 2015; Monteiro et al., 2013; Perentes et al., 2011). As ARF6 activation (GTP binding) involves guanine exchange factors
associated with the plasma membrane (D'Souza-Schorey and Chavrier, 2006; Donaldson and Jackson, 2011), up-regulation of membranous ARF6 in high-grade TNBCs suggests that it is the active form of ARF6 that may accumulate at the plasma membrane in this subset of highly invasive breast tumors coincident with elevated MT1-MMP surface levels.

These data reveal the importance of ARF6 in orchestrating cooperative mechanisms to promote MT1-MMP exocytosis from late endosomal compartments and to drive the invasive potential of TNBC cells. Our findings are integrated in a model shown in Fig. 7FG. MT1-MMP-positive endosomes undergo bidirectional movements along microtubules through opposing activities of dynactin/dynein and kinesin-1 and kinesin-2. Experimentally, normal balance of opposing motors can be perturbed by dynactin or KIFB5 or KIF3A gene silencing (or overexpression) with resulting bias of MT1-MMP endosome movement towards plus or minus end, respectively. Recruitment of KIF5B kinesin-1 and p150\textsuperscript{Glued} dynactin subunits on MT1-MMP-containing vesicles depends on JIP3 and JIP4, in contrast to association of KIF3A kinesin-2 subunit that is JIP independent. JIP4 associates as patches with the cytosolic face of the limiting membrane of MT1-MMP-positive endosomes (localization data for JIP3 are not available). JIP4 patches are closely apposed to and require WASH-positive puncta for endosomal association. Through kinesins, MT1-MMP-positive endosomes reach the cell periphery and the plasma membrane in contact with the ECM. High level of activated GTP-ARF6 at the plasma membrane interacts in trans with JIP3/JIP4 on the endosomal membrane. This scenario is compatible with the crystal structure of GTP-ARF6 bound to the second coiled-coil (LZII) domain of JIP4 (Isabet et al., 2009) (Fig. 7G).
Based on our previous biochemical evidence, binding of GTP-ARF6 to the LZII domain of JIP3/JIP4 dimers occurs preferentially with JIP3/JIP4 bound to dynactin (p150Glued). In contrast, JIP3/JIP4 association with the TPR (tetratricopeptide repeat) domain of kinesin-1 light chain (KLC) may preclude binding of ARF6 to JIP3/JIP4 (Montagnac et al., 2009). We postulate that trans interaction of GTP-ARF6 with JIP3/JIP4-dynactin complex keeps dynein on the leash in a state in which dynein associated with MT1-MMP endosome remains firmly anchored on the microtubule; a situation analogous to the effect of LIS1 acting as a clutch that suppresses dynein movement (Huang et al., 2012; McKenney et al., 2010). In support of this assumption, we observed that loss of ARF6 function correlated with strong dynactin/dynein-dependent perinuclear positioning of MT1-MMP-positive compartments whereby increased dynein processivity is possibly due to JIP3/JIP4 interacting with dynactin as shown recently for cargo adaptors such as Bicaudal or FIP3 (King and Schroer, 2000; McKenney et al., 2014; Tripathy et al., 2014). Several studies have reported how kinesins and dynein/dynactin localized on the same cargo can mechanically compete in a tug-of-war mechanism (reviewed in (Hancock, 2014)). A recent study in particular showed in a quantitative model how endosomes become elongated as a result of the two types of motors pulling in opposite directions (Soppina et al., 2009). Thus we postulate that kinesin-1 possibly pushed by JIP3/JIP4 (Sun et al., 2011), pulls on the membrane of immobilized MT1-MMP-positive endosomes in a tug-a-war with stalled dynein/dynactin leading to tube elongation (Fig. 7F).

We previously reported that MT1-MMP exocytosis at invadopodia involves formation of tubular membrane connection between MT1-MMP-containing LEs and the invadopodial plasma membrane (Monteiro et al., 2013). We found that endosomal
WASH is required for MT1-MMP exocytosis, possibly by controlling actin-based endosomal membrane deformation required for membrane tubulation (Derivery et al., 2012; Gomez et al., 2012; Temkin et al., 2011). Juxtaposition of JIP3/JIP4 and WASH patches on the endosomal membrane would allow optimal coupling between actin-based membrane deformation and pulling force generation through kinesin-1 for membrane tube generation. Kinesin-2, which is not coordinated with dynactin/dynein through ARF6-JIP3/JIP4 cannot substitute for kinesin-1 for tube generation clearly indicating non-redundant functions of endosomal kinesin-1 and -2. Then, fusion of the membrane tube with the invadopodial plasma membrane allows transfer of MT1-MMP to the cell surface (Monteiro et al., 2013; Steffen et al., 2008; Yu et al., 2012). In a final step, GTP-hydrolysis on ARF6 to let dynein off the leash would allow clearance of MT1-MMP-positive endosomes from the cell periphery. This mechanism allows MT1-MMP endosomes to dynamically move and switch direction and provides invasive cancer cells with the necessary plasticity to adapt to changing ECM microenvironments. In conclusion we have delineated the mechanism by which ARF6 through JIP3/JIP4 controls intracellular trafficking and exocytosis of MT1-MMP to promote pericellular matrix remodeling during invasion by TNBCs. Components of the ARF6-JIP3/4-motor axis represent potential important targets in aggressive TNBCs that currently lack targeted therapies.
MATERIALS AND METHODS

Cell culture and antibodies. Human breast adenocarcinoma MDA-MB-231 cells (American Type Culture Collection HTB-26) were maintained in L-15 culture medium (Sigma–Aldrich, St. Louis, MO, USA) with 2 mM glutamine (GIBCO) and 15% FBS (GIBCO) at 37 °C in 1% CO₂. MDA-MB-231 cells stably expressing MT1-MMPmCherry (MT1-MMPmCh) were cultured in the presence of 0.5 mg/mL G418 (Sakurai-Yageta et al., 2008b). MCF10DCIS.com cell line was purchased from Asterand (Detroit, MI) and maintained according to the supplier's guidelines. A list of antibodies used for these studies is provided in Table S3.

DNA constructs, transfection and generation of stable cell lines. Plasmids encoding KIF5B-YFP and KIF3A-GFP have been previously described (Wiesner et al., 2010). JIP3 LZI domain (amino acid 1-266) was obtained by PCR amplification from full-length human JIP3 cDNA (Montagnac et al., 2009). Leucine residues at position 83 and 89 were mutagenized into Proline using the QuikChange II Site-Directed Mutagenesis Kit (Agilent). MDA-MB-231 stably expressing MT1-MMPmCh were transfected using Nucleofector (Lonza) according to the manufacturer’s instructions with 1μg of KIF5B-YFP or KIF3A-GFP plus 100 ng of empty vector pBabe-puro and selected by puromycin (1 μg/ml, GIBCO). Puromycin-resistant cells were sorted for GFP and mCherry expression and amplified in the presence of puromycin.

Indirect immunofluorescence and confocal microscopy. To visualize endogenous motors and JIP4 association to MT1-MMP-positive endosomes, MDA-
MB-231 cells stably expressing MT1-MMPmCh were cultured on gelatin-coated cover-slips, pre-permeabilized with 0.5% Triton-X100 in microtubule stabilizing buffer (MTSB: 4% PFA, 100mM PIPES pH 6.9, 10 μM taxol, 1mM EGTA) for 2 min and then fixed in MTSB for 20 min at 37°C. Then cells were washed in PBS and stained with primary antibodies (see Table S3) and counterstained with appropriate Alexa488- and Cy5-conjugated secondary antibodies (Molecular Probes). For confocal microscopy analysis, image acquisition was performed with A1 R Nikon confocal microscope with a 60x oil objective and a z-dimension series of images was taken every 0.5 μm. Proximity Ligation Assay (PLA) was performed as described (Monteiro et al., 2013).

**Analysis of endosome distribution.** The custom MATLAB-based software to determine the position of MT1-MMP-positive endosomes relative to cell centroid-periphery has been previously described (Castro-Castro et al., 2012).

**Immunohistochemistry analysis of breast cancer tissue microarray.** Analysis of human samples was performed in accordance with French Bioethics Law 2011-814, the French National Institute of Cancer (INCa) Ethics Charter and after approval by Institut Curie Review Board and Ethics committee (Comité de Pilotage du Groupe Sein) that waived the need for written informed consent from the participants. Women were informed of the research use of their tissues and did not declare any opposition for such research. Data were analyzed anonymously. Determination of pathological grade and molecular subtype of breast tumor specimens, tissue microarray (TMA) construction and immunohistochemistry (IHC) labeling of alcohol formalin acetic acid-
fixed paraffin-embedded samples have been already described (Lodillinsky et al., 2015). IHC labeling intensity in carcinoma cells was scored using marker specific 0-to-3 scales (S8A-D Fig) and multiplied by the percentage of positive cells (H-score). Average H-score of duplicate tissue cores was calculated. Distribution of H-score values for each marker according to breast cancer subtype and tumor grade was compared by ANOVA test and plotted using ggplot2 R package. IHC H-scores were scaled and analyzed by unsupervised hierarchical clustering employing the Ward linkage clustering algorithm with euclidean distance as the similarity metric using R Software (version 3.1.1). H-score variables were discretized either in low and high expression in order to perform association test (Table S2). Cut-off values for IHC H-score were calculated using normal mixture modeling (Mclust R package). For MT1-MMP and ARF6, we used membranous H-scores with a threshold estimated to 100. For KIF5B and KIF3A, total H-scores (the sum of membranous and cytoplasmic IHC H-scores) were used and threshold was estimated to 200. H-scores were compared between groups by the chi-square test or Fisher’s exact test when appropriate using R Software. P-values ≤ 0.05 were considered statistically significant.

**Statistics.** Except for IHC data of tumor specimens, statistical analyses were performed using Mann-Whitney t-test, one-way or two-way ANOVA and chi-square test, using GraphPad Prism (GraphPad Software) with p < 0.05 considered significant.

See Supporting Materials and Methods for siRNA treatment and lentiviral vectors for shRNA expression, image deconvolution analysis, gelatin and collagen degradation
assay, MT1-MMPpHLuorin exocytosis assay, TIRF-M and live-cell spinning disk confocal microscopy and immunoprecipitation and immunoblotting analysis.
Dr GM. Griffiths is acknowledged for critical reading of the manuscript. We thank the Breast Cancer Study Group and patients of Institut Curie for breast tumor samples and the Nikon Imaging Centre @ Institut Curie-CNRS and Cell and Tissue Imaging Facility of Institut Curie, member of the France Bio Imaging national research infrastructure (ANR-10-INSB-04) for help with image acquisition. Dr A. Soltani is acknowledged for contribution to pilot experiments, Dr C. Laurent for help with IHC dataset analysis, Dr P. Paul-Gilloteaux for help with endosome position analysis and Dr A. Kawska at IlluScientia.com for artwork. Dr A. Houdusse is acknowledged for help with ARF6/JIP4 3D structure used in Fig. 7G. Drs S. Linder and R.D. Vale are thanked for providing reagents for this study. VM was supported by fellowships from Domaine d'Intérêt Majeur de la Région Ile-de-France and Ligue Nationale contre le Cancer, ACC was supported by a Long Term EMBO fellowship, CL was supported by a postdoctoral fellowship from Fondation Pierre-Gilles de Gennes pour la Recherche, JC by a MD Master 2 fellowship from Institut Curie, EI by a postdoctoral fellowship from Ligue Nationale contre le Cancer and LF and MI by the Incentive and Cooperative Research Programme ‘Breast cancer: cell Invasion and Motility’ of Institut Curie. Funding for this work was provided by grants from ARC (SL220100601356 & SLR20130607099) and Institut National du Cancer (2012-1-PL BIO-02-IC-1) to PC and by core funding from Institut Curie and Centre National pour la Recherche Scientifique (CNRS).
REFERENCES


FIGURE LEGENDS

Figure 1. ARF6 is required for matrix degradation and invasive migration of breast tumor cells through 3D type I collagen. (A) FITC-gelatin degradation (n, number of cells scored for each cell population treated with indicated siRNAs). ***, p < 0.001 (compared with siNT-treated cells). (B) Phalloidin-labeled MDA-MB-231 cell spheroids after two days in 3D collagen I (T2). Insets show spheroids immediately after embedding in collagen (T0). Scale bars: 200 μm. (C) Average invasion area of spheroids at T2 normalized to mean spheroid area at T0 and to invasion of siNT spheroids set to 100 ± SEM (n, spheroids number). ***, p < 0.001. (D) MDA-MB-231 cells treated with indicated siRNAs embedded in collagen I and stained for cleaved collagen with Col1-3/4C antibody (black in the inverted image). DAPI-stained nuclei are shown in red. Scale bar, 20 μm. (E) Collagenolysis by MDA-MB-231 cells (Col1-3/4C antibody signal as in D). Values are mean normalized degradation index ± SEM (n, number of cells analyzed for each cell population).***, p < 0.001 (as compared with siNT-treated cells).

Figure 2. Regulation of MT1-MMP-positive endosome positioning by ARF6 and JIP3/JIP4. (A) JIP4 staining (green) of MT1-MMPmCh-expressing MDA-MB-231 cells (blue). Scale bars, 5 μm, 2 μm (insets at higher magnification). (B-C) Average invasion area of multicellular spheroids after two days in 3D collagen normalized to mean spheroid area at T0 and to invasion of siNT-treated spheroids set to 100 ± SEM (n, spheroids number). ***, p < 0.001. (D) Collagenolysis by MDA-MB-231 cells treated with indicated siRNAs. Values are mean normalized degradation index ± SEM (n, number of cells analyzed for each cell population). *, p < 0.05; ***, p < 0.001.
(as compared with siNT-treated cells). (E) Distribution of MT1-MMPmCh endosomes in MDA-MB-231 cells plated on 2D gelatin. Mean percentage of MT1-MMP-positive endosomes according to their cell center-to-cell periphery position ± SEM. **, p < 0.01; ***, p < 0.001 (compared with siNT distribution). (F) Inverted still images from time-lapse sequences of MDA-MB-231 cells expressing MT1-MMPmCh treated with indicated siRNAs (see Movie S1). Insets show color-coded time projections of selected time frames from these sequences (color code shown on the left, lower row) corresponding to boxed regions at higher magnification. Scale bar, 10 µm. (G) Distribution of MT1-MMPmCh endosomes in MDA-MB-231 cells expressing the indicated JIP3-LZI construct as in panel E. (H) MT1-MMPmCh endosome distribution as in panel E. ns, non-significant, *, p < 0.05; **, p < 0.01; ***, p < 0.001 with siARF6+si150Glued as compared to siARF6 treatment and siJIP3/4+si150Glued as compared to siJIP3/4.

Figure 3. JIP3/JIP4 regulate KIF5B and p150Glued but not KIF3A association to MT1-MMP endosomes. (A-D) Association of indicated motor proteins (green and red) with MT1-MMPmCh positive-endosomes (blue). (E) MDA-MB-231 cells expressing MT1-MMPpHluorin (green) analyzed by in situ proximity ligation assay (PLA, red) with p150Glued and JIP4 antibodies. Scale bars, 2 µm. (F) PLA signal in siRNA-treated cells using indicated antibodies [in brackets]. ***, p < 0.001 (as compared with siNT-treated cells). n, number of cells analyzed for each cell population. (G) MDA-MB-231 cells expressing MT1-MMPmCh treated with indicated siRNAs and stained for KIF5B and p150Glued (upper panels), or KIF5B and KIF3A (lower panels). Scale bars, 2 µm. (H) Motor protein association (green and red signal) with MT1-MMP-positive compartments (blue) compared to association in
siNT-treated control cells (see Materials and Methods section). n, number of endosomes analyzed for each cell population. NS, not significant; *, p < 0.05; **, p < 0.01 (as compared with siNT-treated cells). (I) Detection of KIF5B and KIF3A endogenous proteins in anti-MT1-MMP immunoprecipitates from MDA-MB-231 cells. (J) Detection of MT1-MMPmCh in anti-GFP immunoprecipitates of YFP, KIF5B-YFP or KIF3A-GFP. (K) Detection of KIF5B, p150\textsuperscript{Glued} and KIF3A motor proteins in MT1-MMPmCh immunoprecipitates from MDA-MB-231 cells transfected with indicated siRNAs. (L) Quantification of motor association from immunoblotting analysis as in I. Levels in siNT control cells were set to 100. NS, not significant; *, p<0,05; **, p<0.01 (as compared with siNT-treated cells).

Figure 4. KIF3A mediates peripheral distribution of MT1-MMP endosome in JIP3/JIP4 depleted cells. (A) Silencing of KIF3A and JIP3/4 in MT1-MMPmCh-expressing MDA-MB-231 cells. β-actin was used as a loading control. (B) Inverted still images from time-lapse sequences of MDA-MB-231 cells expressing MT1-MMP-mCherry endosomes treated with indicated siRNAs (see Movie S2). Scale bar 5 µm. (C) Mean percentage distribution of MT1-MMPmCh-positive endosomes according to their cell center-to-cell periphery position ± SEM. ***, p < 0.001 (compared with siNT-treated cells). (D) Color-coded time projections of 61 consecutive time frames from time-lapse sequences of MDA-MB-231 cells expressing MT1-MMPmCh (acquired with 3 s intervals). Scale bar 5 µm. (E, F) Displacement index of MT1-MMP-positive endosomes in indicated cell populations. ***, p < 0.001 (compared with siNT-treated cells in panel E or with DMSO-treated cells, panel F). n, number of scored cells.
Figure 5. KIF5B and KIF3A are required for pericellular matrix remodeling and MT1-MMP exocytosis. (A-C) Silencing of KIF5B and KIF3A and MT1-MMP in MDA-MB-231 cells. β-actin was used as a loading control. (D) FITC-gelatin degradation (n, number of cells scored for each MDA-MB-231 cell population treated with indicated siRNAs). ***, p < 0.001 (compared with siNT-treated cells). (E) Average invasion area of MDA-MB-231 cells using using the circular invasion assay. Values represent mean invasion index ± SEM from three independent experiments normalized to invasion index of siNT-treated cells set to 100. n, number of 96-wells analyzed for each cell population. ***, p < 0.001 (compared with siNT-treated cells). (F) FITC-gelatin degradation by MCF10DICIS.com cells treated with indicated siRNAs as in panel D. (G) Invasion of MCF10DICIS.com cells as in panel E. (H) Collagenolysis by MDA-MB-231 cells treated with indicated siRNAs. Values are mean normalized degradation index ± SEM (n, number of cells analyzed for each cell population). *, p < 0.05; ***, p < 0.001 (as compared with siNT-treated cells). (I) MDA-MB-231 cells expressing MT1-MMPpHluorin silenced for the indicated proteins were seeded on type I collagen fibers and imaged over a 30-min time period. Frequency of MT1-MMPpHluorin exocytic events was quantified (events/cell/min). n, number of cells analyzed for each cell population. ***, p < 0.001 (as compared with siNT-treated cells).

Figure 6. Stimulation of matrix degradation by KIF5B correlates with increased ARF6-, JIP3/4- and p150Glued-dependent endosome tubulogenesis. (A) Inverted still images from time-lapse sequences of MDA-MB-231 cells expressing KIF5B-YFP and MT1-MMPmCh treated with indicated siRNAs (see Movie S3). Scale bar 10 µm. (B) Mean percentage distribution of MT1-MMPmCh-positive endosomes according to
their cell center-to-cell periphery position ± SEM. ***, p < 0.001 (compared with siNT-treated cells). (C) FITC-gelatin degradation by MDA-MB-231 cells overexpressing MT1-MMPmCh together with YFP or KIF5B-YFP. (n, number of cells scored for each MDA-MB-231 cell population treated with indicated siRNAs). ***, p < 0.001 (compared with siNT-treated cells). (D) High magnification galleries of peripheral cell regions (boxed in panel A). Arrows point to tubulated MT1-MMP endosomes. Scale bar, 5 µm. (E) TIRFM images of MDA-MB-231 cells expressing MT1-MMPmCh and KIF5B-YFP plated on gelatin. Gallery shows images with 0.2 sec intervals corresponding to the boxed region. Arrows point to membrane tubulation events. Scale bar, 5 µm. (F, G) Number of tubulated MT1-MMPmCh endosomes per frame normalized for the surface area. Mean ± SEM (n, number of peripheral cell regions scored for each cell population). **, P < 0.01; ***, P < 0.001 as compared to siNT condition.

Figure 7. JIP4 recruitment on MT1-MMP-positive endosomes depends on WASH. (A) JIP4 (green) and cortactin (red) staining of MDA-MB-231 cells expressing MT1-MMP-mCherry (blue) treated with indicated siRNAs. Scale bars, 5 µm, 2 µm (insets at higher magnification). (B) JIP4 (green bars) and cortactin (red bars) association with MT1-MMPmCherry positive-endosomes as in Fig. 3H. ***, p < 0.001 (as compared with siNT-treated cells in which association was set to 100). (C) Inverted still images from time-lapse sequences of MDA-MB-231 cells expressing KIF5B-YFP and MT1-MMPmCh treated with indicated siRNAs. Scale bar, 10 µm. Galleries show images with 30 sec intervals corresponding to the boxed regions. Arrows point to tubulated endosomes. Scale bar, 5 µm. (D) Quantification of tubulation from time-lapse sequences as in panel C. All values are mean ± SEM. n,
number of cell regions scored for each cell population. ***, p < 0.001 (as compared with siNT-treated cells). (E) FITC-gelatin degradation by MDA-MB-231 cells overexpressing MT1-MMPmCh and KIF5B-YFP treated with indicated siRNAs. (n, number of cells scored for each cell population). ***, p < 0.001 (compared with siNT-treated cells). (F) Scheme depicting ARF6 and JIP3/JIP4-mediated regulation of a kinesin-1 and dynactin/dynein tug-of-war leading to MT1-MMP endosome tubulation and surface delivery of MT1-MMP (see text). (G) Model of trans interaction between plasma membrane GTP-ARF6 and endosomal JIP4. ARF6 myristoylated amphipatic N-terminal helix is indicated as a green cylinder lying against the plasma membrane. Only the second coiled-coil domain of JIP4 is shown. Binding of GTP-ARF6 to JIP4 dimer is compatible with dynactin/dynein interaction with JIP4, while it prevents kinesin-1 interaction (based on structural and biochemical data (Isabet et al., 2009; Montagnac et al., 2009).

Figure 8. ARF6 expression increases during breast tumor progression. (A) ARF6 expression in representative regions of in situ (upper row) and invasive components (lower row) of invasive ductal carcinoma (IDC) of the indicated molecular subtypes stained for ARF6 by IHC. LUM, hormone receptor-positive (Luminal A+B); TNBC, hormone receptor-negative, HER2-negative; HER2, hormone receptor-negative, HER2-positive. Scale bars, 10 µm. (B, C) Semi-quantitative analysis of cytosolic (panel C) and membranous ARF6 expression (panel D) by the H-score method comparing peritumoral breast epithelial tissue (n=324), in situ (n=131) and invasive (n=426) components of IDCs. Comparisons were made with Kruskal-Wallis test. NS, non significant, **, P < 0.01; ***, P < 0.001.
Figure 9. Membranous ARF6 and MT1-MMP and KIF5B and KIF3A expression in breast cancer. (A-D) Expression of membranous MT1-MMP (A), membranous ARF6 (B), KIF5B (C) and KIF3A (D) was measured by IHC on TMA. Example of score assignment. (E) Expression of MT1-MMP, ARF6, KIF5B and KIF3A segregated in low and high expression classes is significantly increased in tumor component of IDCs as compared to adjacent peritumoral area. Values are in % of total tumors. (F-G) Correlation of marker expression with molecular subtypes (panel F) and pathological grades (panel G). Analysis was performed on 311 cases for which scores were available for all four markers. ARF6 and MT1-MMP plasma membrane H-scores and total (cytosol + plasma membrane) KIF5B and KIF3A H-scores were considered.

Figure 10. ARF6, MT1-MMP and KIF5B are up-regulated in TNBCs. (A-D) Immunohistochemistry analysis of MT1-MMP, ARF6, KIF5B and KIF3A in invasive ductal carcinomas. Insets show peritumoral area. Scale bars, 25 μm, 10 μm (insets). (E) Hierarchical clustering of the staining patterns of 311 IDC samples based on total KIF3A and KIF5B, and membranous ARF6 and MT1-MMP expression. Data are shown in a table format with vertical axis listing IDC samples and columns representing antibodies. Color scale, which represents relative staining patterns of each sample, is displayed on upper left corner. Molecular subtypes (HER2, TNBC and Luminal A+B) and grades are depicted between clusters and dendrogram with color-coding annotated on the lower left corner.
Marchesin, Castro-Castro et al - Figure 1
Marchesin, Castro-Castro et al - Figure 2
Marchesin, Castro-Castro et al - Figure 4
IDC TMA/anti-ARF6 (IHC analysis)
in situ component

LUM | HER2 | TNBC

invasive component

LUM | HER2 | TNBC

B

---

** | NS
H-score ARF6 (cytosol)

C

---

** | **
H-score ARF6 (membranous)

Marchesin, Castro-Castro et al - Figure 8